Section II (Remarks)

Summary of Amendment to the Claims

By the present Amendment, claims 1, 3-8, 10, 13-15, 18, 21, 46, 48 and 49 have been amended. New claim 62 has been added and is supported in the specification, as originally filed at page 18, lines 22-24. No new matter within the meaning of 35 U.S.C. §132(a) has been introduced by the foregoing amendments.

The amendments do not require a new search, or raise new issues for consideration because they merely address issues already raised by the examiner or define applicant's invention more clearly. It is submitted that the amendments place the claims in condition for allowance or in better condition for appeal by reducing the number of issues for consideration on appeal. The amendments were not made earlier in the prosecution because it is maintained that the previously pending claims were allowable.

The amendments made herein are fully consistent with and supported by the originally-filed disclosure of this application. Entry of the Response into the file of the application is respectfully requested.

Thus, upon entry of the amendments, claims 1-62 will be pending, of which claims 18-45 and 50-61 are withdrawn.

Objection to the Specification

In accordance with the examiner's request, claim 10 has been amended to include reference to the sequence identifier "SEQ ID NO: 7." The amended application, including the claims, is in compliance with the requirements set forth in 37 C.F.R. §1.821(a)(2) for applications containing amino acid sequences.

Since the correction requested by the examiner has been made in the present Response, withdrawal of the objection is requested.

Information Disclosure Statement

It is acknowledged that a copy of the 1993 Strausberg et al. reference (described by the examiner as the 1995 reference) was not provided with the Information Disclosure Statement mailed

October 13, 2008. The examiner's direction of applicant's attention to this oversight is appreciated.

A copy of the reference is attached hereto as Appendix A. As noted by the examiner, this reference was included by citation in the IDS filed October 13, 2008. Entry of this reference into the file history of the application is respectfully requested.

The examiner provides that "Applicant's Remarks, at page 23 [of the Response mailed August 12, 2008], supply sufficient information for citing Strausberg et al., 2005…on…Form PTO-892." Entry of this reference into the record of the application is noted, however, it is also noted that the priority date of the present application is August 6, 2003. Strausberg et al. 2005 was cited in the Response mailed August 12, 2008 to support attorney arguments made in that Response. Strausberg et al. 2005 is not eligible as, nor should it be considered as, prior art to the present application.

Rejection of Claims Under 35 U.S.C. § 112

Claims 3, 7, and 13 are rejected under 35 U.S.C. §112, second paragraph as indefinite for recitation of the phrase "…prodomain is modified to bind subtilisin or a variant thereof with increased affinity as compared to an unmodified prodomain protein…" in each of such claims.

Neither of claims 3 or 7 contains the phrase as specifically recited by the examiner. Claim 3 recites a proviso that "… the protease prodomain protein further comprises one or more amino acid substitutions that increase binding affinity for subtilisin or a variant thereof…", and claim 7 recites "…the protease prodomain protein is modified to exhibit an increased affinity for subtilisin or a variant thereof…" The language of claim 13 is slightly different from that cited by the examiner, where claim 13 recites "…the protease prodomain protein is modified to bind subtilisin or a variant thereof with increased affinity as compared to an unmodified protease prodomain protein" (emphasis added).

With regard to the subtilisin element of each claim, the examiner alleges that "no particular subtilisin is identified…that would permit the artisan and the public…to differentiate any particular subtilisin from a 'variant thereof.'" Applicant respectfully disagrees and traverses the rejection.

It is well established that "[d]uring patent examination, the pending claims must be given the broadest reasonable interpretation consistent with the specification. *In re Morris*, 127 F.3d 1048, 1054, 44 USPQ2d 1023, 1027 (Fed. Cir. 1997); *In re Prater*, 415 F.2d 1393, 162 USPQ 541 (CCPA 1969)." (MPEP §2173.05). The examiner's attention is respectfully drawn to the specification at page 3, lines 1-14, where subtilisin is described in detail. The structural aspects of subtilisin are described at page 4, lines 1-28. Subtilisin is a protease produced by numerous species of bacteria or fungi and is not limited to a specific sequence. As described in the specification "numerous subtilisins are known and include …subtilisin BPN, subtilisin Carlsberg, subtilisin DY, subtilisin anylosacchariticus, and mesenticopeptidase." (Specification, p. 3) One of skill in the art, based on this description and the knowledge in the art would be able to identify, without undue experimentation, a variety of subtilisins for use in binding to a protein substrate encoded by the construct of claim 3, for use in binding to the fusion protein of claim 7 or useful in the method of claim 13.

Similarly, various mutations of subtilisin were known in the art at the time of filing of the present application, as described in the specification at page 3, lines 9-14. The terms "mutation," "variant" and "subtilisin-like proteases" are all defined in the application at pages 13-14.

The examiner's attention is respectfully drawn to Appendix B hereto, a printout from the "Structural Classification of Proteins" website located at hyper text transfer protocol web address: scop.mrc-lmb.cam.ac.uk/scop/data/scop.b.d.fg.b.html. This printout shows classification of the subtilisin-like superfamily and characteristics thereof. It is well established that subtilisin-like proteases have a widely recognized and consistent mechanistic and structural definition that is known and recognized by those of skill in the art.

The examiner's attention is respectfully drawn to MPEP § 2173.04 "[b]readth of a claim is not to be equated with indefiniteness. *In re Miller*, 441 F.2d 689, 169 USPQ 597 (CCPA 1971). If the scope of the subject matter embraced by the claims is clear, and if applicants have not otherwise indicated that they intend the invention to be of a scope different from that defined in the claims, then the claims comply with 35 U.S.C. 112, second paragraph."

While the class of subtilisins and variants thereof may be a broad class of proteases, such a class is not necessarily indefinite by virtue of its size. From the description in the specification and with the knowledge in the art at the time of filing of the application, one of skill in the art would be able to identify both a subtilisin and/or a variant of a subtilisin, as claimed, without undue

experimentation.

The examiner has also alleged that "a prodomain protein further comprising one or more amino acid substitutions according to the preceding recitations in each claim cannot be distinguished by the artisan and the public…from a 'compar[ison] prodomain portion with no substitutions.'" It is the examiner's position that "the absence of any structure defining a starting point for determining what is, and what is not, a prodomain with no substitutions leaves the scope of claims 3, 7 and 13 indeterminate and indefinite." (Final Office Action, p. 3.) Applicant respectfully disagrees and traverses the rejection.

The examiner's attention is respectfully drawn to the amended claims as set forth in Section I above, where the language "prodomain protein" has been amended to a "protease prodomain protein" throughout the claims. Protease prodomain proteins, in particular subtilisin prodomain proteins are described in detail throughout the specification and are particularly defined at page 14, line 31 to page 15, line 6:

> The term "protease prodomain protein" refers to prodomain amino acid sequence or functional equivalent thereof wherein the protease prodomain protein possesses the capability of binding to a corresponding protease with high affinity. Preferably, the prodomain is substantially free of other proteins with which it is naturally associated, for instance, the balance of the protease protein. In addition, one or more predetermined amino acid residues in the prodomain may be substituted, inserted, or deleted, for example, to produce a prodomain protein having improved biological properties, or to vary binding and expression levels. Through the use of recombinant DNA technology, the prodomain proteins of the present invention having residue deletions, substitutions and/or insertions may be prepared by altering the underlying nucleic acid.

Furthermore, the protease prodomain proteins recited in claims 3, 7 and 13 are not simply described by their structural characteristics. In each claim, the modified prodomain protein also possesses the functional characteristic of increased binding affinity as a substrate for subtilisin or a variant thereof. When a person of skill in the art has modified the "protease prodomain protein" by substitution, insertion or deletion, that person already has a starting protein. Attempting various substitutions, insertions or deletions and comparing the affinity of the resulting modified protease prodomain protein with the starting, unmodified protease prodomain protein is well within the skill of one in the art.

From the description in the specification and with the knowledge in the art at the time of filing of the application, one of skill in the art would be able to generate a modified protease prodomain

protein with increased binding affinity as compared to the starting product, an unmodified protease prodomain protein, in order to determine increased affinity for subtilisin or a variant thereof, as claimed.

Withdrawal of the rejection of claims 3, 7, and 13 under 35 U.S.C. §112, second paragraph as indefinite is respectfully requested.

Rejection of Claims Under 35 U.S.C. § 102

In the Office Action mailed December 31, 2008, the examiner has maintained the rejection of claims 1, 46 and 47 under 35 U.S.C. §102(e) as anticipated by U.S. Patent Publication No. 2003/0166162, hereinafter referred to as "Van Rooijen et al." Applicant respectfully disagrees and maintains traversal of the rejection.

Both of independent claims 1 and 46 (and claim 47, which depends from claim 46), recite a portion of the claimed nucleic acid construct that encodes a protein (a protease prodomain protein in claim 1) that binds with high affinity to a protease or a variant thereof. Van Rooijen et al. does not provide a nucleic acid construct encoding protein portions with the characteristics of the prodomains recited in the claims of the present application, namely, with high binding affinity for a protease or variant thereof.

It was previously detailed in the Response mailed August 12, 2008 that the constructs of Van Rooijen et al. do not show high binding affinity to chymosin. The "affinity" of a protein for a protease, as known in the art, is the ability to bind to the protease and stay bound. High binding affinity is evidenced by a low disassociation constant, as the elements remain bound to one another and do not disassociate.

As evidence of these characteristics of a protein with high binding affinity, Applicant directed the examiner's attention to the specification at page 18:

> "[t]he phrase 'binding with high affinity' as used herein refers to the ability of the protease prodomain to bind to the cognate protease with a Kd of nM to pM and ranging from about 10 nM to about 10 pM, preferably < 100 pM."

In response, the examiner stated that "were [the definition of high binding affinity] to be stated verbatim in a claim, [it] would be indefinite." However, applicant respectfully submits that this

definition is not set forth as a claim. The definition is provided to support the recitation of "high affinity" in independent claims 1 and 46.

It is well established that "[d]uring examination, the claims must be interpreted as broadly as their terms reasonably allow. *In re American Academy of Science Tech Center*, 367 F.3d 1359, 1369, 70 USPQ2d 1827, 1834 (Fed. Cir. 2004)" and that "[t]his means that the words of the claim must be given their plain meaning unless the plain meaning is inconsistent with the specification. *In re Zletz*, 893 F.2d 319, 321, 13 USPQ2d 1320, 1322 (Fed. Cir. 1989)…; *Chef America, Inc. v. Lamb-Weston, Inc.*, 358 F.3d 1371, 1372, 69 USPQ2d 1857 (Fed. Cir. 2004)." (MPEP §2111.01) Direction of the examiner's attention to the definition of "binding with high affinity" at page 18 of the specification is performed in order to demonstrate that applicant's use of the phrase "high affinity" is in accordance with the plain meaning of that phrase.

Therefore, the invention of claims 1, 46 and 47 is nucleic acid constructs, and each must contain a portion which is a protein (a protease prodomain protein in claim 1) that binds with high affinity to a protease or a variant thereof.

The examiner indicated that the claims do not "indicate any particular structures conferring the stated degree of affinity…" Applicant respectfully disagrees. Independent claims 1 and 46 contain both structural and functional requirements.

In claim 1 the nucleic acid construct encodes a fusion protein that structurally comprises the elements 1) coding sequence for a protein of interest and 2) coding sequence for a protease prodomain protein, where the fusion protein functionally has the two elements operatively linked and where, functionally, the protease prodomain protein binds a protease or a variant thereof with high affinity.

In claim 46 the nucleic acid construct encodes a fusion protein that structurally comprises elements 1) coding sequence for a protein of interest and 2) coding sequence for a second protein, where the second protein binds a protease or a variant thereof with high affinity. Claim 47 depends from claim 46 and therefore, by virtue of its dependency, must contain all limitations recited in claim 46.

In addition to the definition known in the art and the corroborating definition provided in the specification, the examples set forth in the specification support this definition of binding with

high affinity. The examples illustrate purification using the claimed constructs, where a protein of interest is linked to a prodomain (or second protein) and the prodomain binds a protease tightly. Then the bound prodomain-protease and the protein of interest are cleaved from one another. The protein of interest is thus recoverable and the bound prodomain is disabled, as it remains bound to the protease after cleavage. Therefore "affinity" as used in claims 1 and 46 describe a prodomain protein that can bind tightly and remain bound to a protease.

It is undisputed that Van Rooijen et al. provide a polynucleotide encoding a fusion polypeptide, comprising a target protein fused to a propeptide. However, Van Rooijen et al. provide a construct encoding a fusion polypeptide that is both structurally and functionally different from the fusion proteins produced from the presently claimed constructs of claims 1, 46 and 47.

In Example 4 of Van Rooijen et al., the results of which are provided in Table 2, cited by the examiner, a GST-cystatin fusion protein is generated, with chymosin propeptides inserted in various locations to form different fusion proteins (shown in Figs. 13-17: GST-KLIP4-Cystatin, GST-KLIP11-Cystatin, GST-KLIP12-Cystatin, GST-KLIP14-Cystatin, GST-KLIP15-Cystatin, and GST-KLIP16-Cystatin). "Table 2 summarizes the cleavage results obtained for each of the corresponding KLIP peptides summarized in FIGS. 13 through 18 expressed in *E. coli*. as GST-KLIP-Cystatin fusion proteins…[p]olypeptide bands corresponding to the molecular mass of free cystatin were subjected to N-terminal sequencing to determine the precise location of the scissile bond…all KLIP-Cystatin fusions were accurately cleaved…" (para. [0133]-[0134]; emphasis added).

Chymosin is added to each of the fusion proteins and cleaves the fusion protein at the propeptide sites. The chymosin does not bind to the KLIP propeptide with high affinity, but associates only transiently as is typical of enzyme-substrate-product interactions. This allows release of chymosin after cleavage, where it is free to cleave additional fusion proteins. The results of this action are what are shown in Table 2. It is clear from the results (with the exception of KLIP12) that the longer the reaction is allowed to proceed, the chymosin continues to react with additional fusion peptides in the solution and more free cystatin is generated.

Therefore the results shown in Table 2 of Van Rooijen et al. demonstrate the "catalytic turnover" of the fusion protein generated, and do not show binding affinity.

"Catalytic turnover" and "binding affinity" are distinctly different properties. Enzymatic

reactions are commonly characterized using parameters termed "k_{cat}" and "K_M" in the art. k_{cat} is the overall catalytic rate of an enzyme, calculated as the number of substrate molecules converted to product by each catalytic site as a function of time, and is also referred to as "catalytic turnover." K_M, known as the Michaelis-Menten constant, is often (sometimes incorrectly) associated with the affinity of the enzyme for substrate.

Catalytic turnover quantifies the results of the reaction between KLIP and chymosin. Table 2 shows the amount of free cystatin generated as multiple reactions are allowed to occur between the fusion proteins (at the KLIP site) and a determinate amount of chymosin. Van Rooijen et al. do not address the affinity between KLIP and the chymosin. As described above and as utilized in the claims, affinity is a measure of the binding of two components.

To further illustrate the distinction between binding affinity and catalytic turnover, applicant provides the following example of a minimum realistic mechanism for proteolysis:

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} EP_1+P_2 \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} E+P_1$$

where E is enzyme, S is substrate, P_1 is the N-terminal portion of the cleaved protein and P_2 is the C-terminal portion of the cleaved protein. As represented above, S is a fusion protein comprising a protein of interest operatively linked to a protease prodomain, P_1 is the prodomain after cleavage, and P_2 is the protein of interest after cleavage. The rate constant k_2 is the first chemical step in the reaction (i.e. acylation). The unambiguous definition of substrate affinity of the fusion protein is provided by the substrate dissociation constant:

$$K_S = k_{-1} / k_1$$

The unambiguous definition of the product affinity for the prodomain after cleavage is defined by the product dissociation constant:

$$K_{P_1} = k_3 / k_{-3}$$

It frequently is assumed for proteases that $k_{cat} \sim k_2$ and that the $K_M \sim K_S$. Both assumptions are incorrect, however, when either substrate affinity or product affinity are high. This can be seen by examining the actual definitions of k_{cat} and K_M for the mechanism above:

$$k_{cat} = k_2 k_3 / (k_2 + k_3)$$

$$K_M = (k_2 + k_{-1}) k_3 / k_1 (k_2 + k_3)$$

For typical proteolytic reactions (such as those described in Van Rooijen et al. and Grøn et al.),

associations of substrate and product with the protease are transient. That is, the substrate off rate, k_{-1} (ES → E+S) and the product off rate, k_3 ($EP_1+P_2 \rightarrow E+P_1$) are large compared the chemical step k_2 ($ES \rightarrow EP_1+P_2$). When these conditions hold, $(k_2+ k_3) \sim k_3$ and $(k_2+ k_{-1}) \sim k_{-1}$. Thus, for typical proteolytic reactions (such as those described in Van Rooijen et al. and Grøn et al.), $k_{cat} \sim k_2$ and $K_M \sim K_S$.

However, for a proteolytic reaction mediated by a high affinity prodomain, as claimed, the substrate off rate, k_{-1}, and the product off rate, k_3, are small compared the chemical step k_2. In this case, $(k_2+ k_3) \sim k_2$ and $(k_2+ k_{-1}) \sim k_2$. Thus $k_{cat} \sim k_3$ and $K_M \sim k_3/k_1$. Specifically, when substrate and product dissociation constants are $\leq$ 10 nM, both substrate and product dissociation rates fall below 0.01 sec^{-1}. In this case, the K_M is indeed very low (reflecting saturation of the Michaelis complex), but the k_{cat} is very low as well ($k_{cat} < 0.01$ sec^{-1}). In fact, once k_3 falls below k_2, the higher the affinity of the prodomain for the protease, the lower the k_{cat}.

As is clear from the above, k_{cat}, K_M , K_S and K_P are distinctly different calculations. None are interchangeable. In fact, steady-state measurement of k_{cat} and K_M in does not provide any direct information about of the value of either K_S or K_P in a proteolytic reaction mediated by a high affinity prodomain.

Van Rooijen et al. do not provide a showing of a construct encoding a fusion protein comprising a prodomain protein with a high affinity for a protease. In fact, high affinity for subtilisin and other proteases leads to substrate inhibition and poor turnover in the processing of fusion proteins. Very high affinity, as claimed, leads to only a single turnover, due to the continued binding of the protease and the prodomain portion (or the second protein, in claims 46 and 47).

While not recited in claims 1 and 46-47, the fusion proteins encoded by the claimed constructs are useful in methods of protein purification, as is repeated throughout the application, *see* title of the application, Abstract, page 1, lines 9-12, page 3, lines 24-28, page 12, lines 34-36, page 14, lines 11-15, page 15, lines 15-20 and the Examples.

By contrast, the fusion proteins produced in Van Rooijen et al. cannot be used in purification methods. Van Rooijen et al. simply cleave the fusion protein into KLIP and Cystatin and detect a cleaved band of free Cystatin on a gel to determine where such cleavage occurred. Nothing new is provided by Van Rooijen et al. Inclusion of the propeptide with the target protein simply

directs processing the way any protease recognition sequence would. This has been shown before in the art. (*See*, for example, Nakayama et al., *J. Biotechnol.* 1992 Mar; 23(1):55-69; reporting processing of the prepropeptide portions of a protease fused to hGH.)

As Van Rooijen et al. do not describe a nucleic acid construct as set forth in claims 1, 46 or 47, Van Rooijen et al. do not anticipate the claimed invention. Accordingly, withdrawal of the rejection of claims 1, 46, and 47 under 35 U.S.C. § 102(e) as being anticipated by Van Rooijen et al. is respectfully requested.

Rejection of Claims Under 35 U.S.C. § 103

In the Office Action mailed December 31, 2008, the examiner has maintained the rejection of claims 2, 5, 7, 8, 11, 12, 13, 14, 17, and 48 under 35 U.S.C. §103(a) as obvious over Van Rooijen et al. in view of Grøn et al., "Studies of binding sites in the subtilisin from *Bacillus lentus* by means of site directed mutagenesis and kinetic investigations," *Subtilisin Enzymes: Practical Protein Engineering*, Bott, R. and Betzel, C., Eds., p. 105-112, 1996 (hereinafter Grøn et al. 1996). Applicant respectfully disagrees and traverses such rejection.

In the Office Action mailed May 13, 2008 claim 3 was included in such rejection. As claim 3 has not been expressly noted as having overcome the rejection as obvious over Van Rooijen et al. in view of Grøn et al. 1996, it is assumed that omission of claim 3 in this list was an unintentional oversight. Discussion of claim 3 as non-obvious over Van Rooijen et al. in view of Grøn et al. 1996 is included herein.

Claims 2, 3, 5, 7, 8, 11, 12, 13, 14, 17, and 48 recite nucleic acid constructs, fusion proteins and methods of using the same. All of these claims, either directly or through dependency on another claim, recite an element of a fusion protein comprising a prodomain protein with a high affinity for a protease. The combination of Van Rooijen et al. and Grøn et al. 1996 does not disclose or describe such a fusion protein.

As discussed above k_{cat} and K_M, are steady state catalytic parameters which can be incorrectly interpreted when substrate and product off-rates are slow (i.e. substrate and product affinity is high). Likewise, the ratio of k_{cat} to K_M is commonly used to provide a measure of enzyme efficiency (or "catalytic efficiency"). For the minimal protease mechanism defined above:

$$k_{cat}/K_M = k_1k_2 / (k_2 + k_{-1})$$

For high substrate affinity $(k_2 + k_{-1}) \sim k_2$ and the reaction approaches the diffusion limit defined by k_1, with all binding events resulting in cleavage. The expression is misleading when product affinity is high, however, because it masks the fact that the enzyme only performs one catalytic cycle and then stops due to product inhibition. While the expression is mathematically correct, it has no physical relevance with regard to a catalytic reaction.

It is well established that affinity, enzyme efficiency and catalytic turnover are separate considerations and are not interchangeable determinations. (*See* Strausberg, S.L. et al. "Directed Coevolution of Stability and Catalytic Activity in Calcium-free Subtilisin," *Biochemistry*, 2005 Mar 8;44(9):3272-9; Hedstrom, L. (2002) "Serine protease mechanism and specificity." *Chem. Rev.* 102, 4501-4524; provided as Exhibits A and B to Response filed August 12, 2008.)

In the claimed invention of the present application, the protease has a very low K_M (reflecting high saturation of the enzyme-substrate complex). The protease captures an equal molar amount of fusion protein and then performs one catalytic cycle and stops. The enzyme and product do not disassociate due to the high affinity of the protease for the prodomain, and the protease is not available to continue to catalyze additional reactions. Thus high prodomain affinity leads to product inhibition and essentially a single turnover. The apparent enzyme efficiency (k_{cat}/K_M) is high because virtually every binding event results in a cleavage event. As noted above, however, the k_{cat}/K_M value has no physical relevance in the claimed invention because each protease molecule binds and converts only a single substrate molecule to product.

As set forth in detail above with respect to the rejection based on 35 U.S.C. §102, it is respectfully submitted that Van Rooijen et al. do not provide a showing of a construct encoding a fusion protein comprising a prodomain protein with a high affinity for a protease. Clearly Van Rooijen et al. provide a measure of free cystatin accumulation as the enzyme chymosin binds to the KLIP peptide and the cystatin is cleaved. Due to the increased cleavage over time, it is apparent that the KLIP and chymosin disassociate and the reaction proceeds. The showing of free cystatin accumulation is a measure of k_{cat}, or continual turnover as the reaction proceeds. As the enzyme binds, releases and rebinds, the reaction proceeds until all substrate is converted (cleaved).

In the rejection under 35 U.S.C. §103, the examiner alleges that "…Van Rooijen et al. teach modifications of a chymosin prodomain that provides a higher affinity for chymosin recognition

in a fusion polypeptide than in a fusion polypeptide comprising a hormone and an unmodified protease prodomain…" Applicant respectfully disagrees.

The examiner's attention is respectfully drawn to para. [0089] of Van Rooijen et al.:

> "[m]utated forms of the pro-peptide may be used to obtain specific cleavage between the propeptide and a heterologous protein. Mutations in the pro-peptide could alter the optimal conditions, such as temperature, pH and salt concentration, under which cleavage of a heterologous protein is achieved…"

and to para. [0090] of Van Rooijen et al., "[t]he purpose of mutating the chymosin pro-peptides was to test to [sic] robustness and flexibility of the system." Nowhere in Van Rooijen et al. is modification of the pro-peptide to increase affinity for chymosin provided.

It is maintained that Van Rooijen et al. do not provide a showing of a construct encoding a fusion protein comprising a prodomain protein with a high affinity for a protease. Combination of Van Rooijen et al. with Grøn et al. 1996 does not remedy this deficiency.

The examiner cites Grøn et al. 1996 as teaching

> "improving the affinity of a subtilisin modified to better accept the S4 amino acid of a subtilisin prodomain's carboxyl terminal region introduced in modified peptide substrates that represent the P4-P3-P2-P1 peptide of the 'corresponding' unmodified prodomain, using nucleic acid constructs encoding the modified subtilisin and encoding the modified subtilisin prodomain, wherein the P4 position is modified by introducing phenylalanine…" (Final Office Action mailed December 31, 2008, p. 5.)

Applicant respectfully disagrees with this characterization of Grøn et al. 1996.

Grøn et al. 1996 does not show affinity changes. In Grøn et al. 1996 "[e]ach subsite was characterized by its substrate preference as determined by the k_{cat}/K_M values." (p. 107). As detailed above, k_{cat}/K_M is a measure of enzyme efficiency which reflects the substrate's ability to participate in a reaction and produce product in a multiple turn-over reaction. It is not a measure of affinity, which reflects the tendency of the reactants to bind and stay bound.

Where Grøn et al. 1996 suggests modification of subtilisin, modifications are provided at various positions S_5, S_4, S_3, S_2, S_1, S'_1, S'_2,and/or S'_3 (p. 106). Each of the different substitutions or combination of substitutions was indicated to represent "different properties of side chains" (p. 106).

The examiner's attention is respectfully drawn to Hedstrom (cited above), where it is stated that

"[m]ost remote interactions do not increase substrate affinity but contribute exclusively to catalysis. This observation suggests that these interactions introduce strain into the substrate, which penalizes binding affinity but accelerates catalysis." (Hedstrom, p. 4517.) Such remote interactions have different effects on each of affinity and catalysis. Accordingly, a showing of a change in k_{cat}/K_M values is not necessarily predictive or correlative to a change in affinity.

Van Rooijen et al. and Grøn et al. 1996 fail to provide any derivative basis for the claimed invention. In combination, the two references fail to provide a showing of fusion protein comprising a prodomain protein with a high affinity for a protease. Accordingly, no basis of *prima facie* obviousness of the claimed invention is presented by such cited references.

As Van Rooijen et al. in view of Grøn et al. 1996 does not provide any logical basis for the constructs, fusion proteins, or methods recited in claims 2, 3, 5, 7, 8, 11, 12, 13, 14, 17, and 48, Van Rooijen et al. in view of Grøn et al. 1996 does not render the claimed invention obvious. Accordingly, withdrawal of the rejection of claims 2, 3, 5, 7, 8, 11, 12, 13, 14, 17, and 48 under 35 U.S.C. § 103 (a) as being obvious over Van Rooijen et al. in view of Grøn et al. 1996 is respectfully requested.

Furthermore, in the Office Action mailed December 31, 2008, the examiner has maintained the rejection of claims 4, 6, 15 and 16 under 35 U.S.C. §103(a) as obvious over Van Rooijen et al. in view of Grøn et al. 1996 and further in view of Grøn et al., *Biochem.*; 1992, 31, 6011-6018 (hereinafter "Grøn et al. 1992"). Claim 49 has further been added to this rejection. Applicant respectfully disagrees and traverses the rejection.

Claims 4, 6, 15, 16 and 49 all recite constructs, fusion proteins, or methods comprising a fusion protein comprising a prodomain protein with a high affinity for a protease where modifications have been made in the protease at one or more of positions P_4, P_3, P_2, and/or P_1. As set forth in detail above, the combination of Van Rooijen et al. and Grøn et al. 1996 fail to provide a showing of fusion protein comprising a prodomain protein with a high affinity for a protease. Further inclusion of Grøn et al. 1996 in the combination of references, fails to render the claimed invention obvious under 35 U.S.C. §103.

It is the examiner's position that combination of Van Rooijen et al. in view of Grøn et al. 1996 and further in view of Grøn et al. 1992 demonstrates constructs, fusion proteins, or methods as recited in claims 4, 6, 15, 16 and 49. With regard to the specific protease modifications, the

examiner asserts that Grøn et al. 1992 shows

> "that the commercially prominent subtilisins, BPN' and Savinase, share a highest binding affinity for phenylalanine at their S4 binding sites, have broad specificity for any amino acid at their S3 binding subsites with preference for a positively-charged amino acid, share a highest binding affinity for alanine at their S2 subsites, and share highest binding affinities for phenylalanine and leucine at their S4 subsites." (Final Office Action mailed December 31, 2008, p. 5.)

In support of this position the examiner directs applicant's attention to "Tables II and III and accompanying discussion spanning pages 6014-6016." (Final Office Action mailed December 31, 2008, p. 5.)

As in the Grøn et al. 1996 reference, the results reported in Tables II and III of the Grøn et al. 1992 reference provide k_{cat}/K_M values and the reference does not show affinity between a prodomain and a protease. Specifically, Table II is titled "k_{cat}/K_M Values for the Cleavage of Fluorogenic Peptide Substrates by Subtilisin BPN' and Savinase and the Associated $\Delta\Delta G_T^{\ddagger}$(Xaa→Gly) Values." $\Delta\Delta G_T^{\ddagger}$ is described in Grøn et al. 1992 as "the activation energy for the enzymatically catalyzed transition of a substrate into product(s)" (p. 6013). Neither k_{cat}/K_M values, nor $\Delta\Delta G_T^{\ddagger}$(Xaa→Gly) values show binding affinity. Table III also shows calculated $\Delta\Delta G_T^{\ddagger}$ values, from which substrate preference is determined. However, substrate preference is not the same as binding affinity. "The properties of the amino acid residues which constitute a given binding subsite determine which amino acid residue(s) of the substrate may bind, and thus, they provide the basis of subsite specificity or preference…" (p. 6011). Substrate preference differs from binding affinity, which reflects the tendency of the substrate and enzyme to bind and stay bound.

The combination of Van Rooijen et al., Grøn et al. 1996 and Grøn et al. 1992 fail to provide any derivative basis for the claimed invention. In combination, the two references fail to provide a showing of fusion protein comprising a prodompain protein with a high affinity for a protease. Accordingly, no basis of *prima facie* obviousness of the claimed invention is presented by such cited references.

As Van Rooijen et al. in view of Grøn et al. 1996 and further in view of Grøn et al. 1992 does not provide any logical basis for the constructs, fusion proteins, or methods recited in claims 4, 6, 15, 16 and 49, Van Rooijen et al. in view of Grøn et al. 1996 and further in view of Grøn et al. 1992 does not render the claimed invention obvious. Accordingly, withdrawal of the rejection of claims 4, 6, 15, 16 and 49 under 35 U.S.C. § 103 (a) as being obvious over Van Rooijen et al. in

view of Grøn et al. 1996 and further in view of Grøn et al. 1992 is respectfully requested.

CONCLUSION

Based on the foregoing, all of Applicant's pending claims 1-17, 46-49 and 62 are patentably distinguished over the art, and in form and condition for allowance. The examiner is requested to favorably consider the foregoing, and to responsively issue a Notice of Allowance.

No fees are believed to be due for the filing of this paper. However, should any fees be required or an overpayment of fees made, please debit or credit our Deposit Account No. 08-3284, as necessary.

If any issues require further resolution, the examiner is requested to contact the undersigned attorney at (919) 419-9350 to discuss same.

Respectfully submitted,

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Encl.
Appendix A [9 pgs.]
Appendix B [2 pgs.]

The USPTO is hereby authorized to charge any deficiency or credit any overpayment of fees properly payable for this document to Deposit Account No. 08-3284

APPENDIX A

Catalysis of a Protein Folding Reaction: Thermodynamic and Kinetic Analysis of Subtilisin BPN′ Interactions with Its Propeptide Fragment†

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ABSTRACT: The in vivo folding of subtilisin is dependent on a 77 amino acid propeptide, which is eventually cleaved from the N-terminus of subtilisin to create the 275 amino acid mature form of the enzyme (Ikemura et al., 1987). We have cloned, expressed, and purified large quantities of the 77 amino acid subtilisin propeptide. This has enabled us to characterize its participation in the subtilisin folding reaction by spectroscopic and microcalorimetric methods. Unfolded subtilisin, when returned to native conditions, is kinetically isolated from its native state. Folding of subtilisin with the native calcium site-A is extremely slow even in the presence of a high concentration of isolated propeptide. The folding of a calcium-free mutant subtilisin, however, is readily catalyzed by the isolated propeptide. The propeptide–subtilisin folding reaction can be described as the following equilibrium: $P_u + S_u \Leftrightarrow P\text{–}S \Leftrightarrow P_f\text{–}S_f \Leftrightarrow P_u + S_f$, where S_u and P_u are subtilisin and propeptide, respectively, which are largely unstructured at the start of the reaction; P–S is a collision complex of unfolded subtilisin and propeptide; P_f–S_f is the complex of folded subtilisin and propeptide; and S_f is folded subtilisin. The rate-limiting step in the folding reaction of calcium-free mutant subtilisin is formation of the initial collision complex, P–S. The rate at which P_u and S_u form a productive collision complex is $\sim$500 M^{-1} s^{-1}. The collision complex appears to be an early folding unit which, once formed, results in rapid isomerization to the fully folded complex. The rate constant for isomerization of the collision complex to the folded complex is $\geq$0.5 s^{-1}. Once folded, propeptide and subtilisin form a tight complex with a K_a of 2×10^8 M^{-1} at 25 °C. The energetics of binding the propeptide to the folded forms of either calcium-free subtilisin or subtilisin with the native calcium A-site are similar and are typical of the folding reaction of a small globular protein.

The *Bacillus* serine protease subtilisin is an unusual example of a monomeric protein with a high kinetic barrier to folding and unfolding (Bryan et al., 1992). Biosynthesis of subtilisin is dependent on a 77 amino acid propeptide, which is eventually cleaved from the N-terminus of subtilisin to create the 275 amino acid mature form of the enzyme (Ikemura et al., 1987). The probable role of the propeptide in subtilisin folding was recently illuminated by analogy with another extracellular microbial protease, α-lytic protease from *Lysobacter* (Baker et al., 1992b). Both subtilisin and α-lytic protease are extracellular, bacterial, serine proteases, though they are not evolutionarily related. α-Lytic protease has a 166 amino acid propeptide which has been shown to catalyze folding as a fusion protein with glutathione transferase (Baker et al., 1992a). As in subtilisin, the folded and unfolded forms of α-lytic protease are separated by a high kinetic barrier. The propeptides of α-lytic protease and subtilisin are strong competitive inhibitors of their respective enzymes (Baker et al., 1992a; Zhu et al., 1989). The propeptide of α-lytic protease has been proposed by Baker et al. to bind to, and therefore stabilize, a native-like transition state in the folding reaction. The 77 amino acid propeptide of subtilisin has been shown to promote subtilisin folding, and mutations have been identified which abolish its function in vivo (Kobayashi & Inouye, 1992). The role of these propeptides in faciliating protein folding is different from the Gro E-type chaperons in two major respects. First, propeptides appear to promote folding by accelerating the folding reaction rather than preventing competing off-pathway reactions (e.g., aggregation). Second, the propeptides are protein specific in their function.

We have cloned, expressed, and purified large quantities of the 77 amino acid subtilisin propeptide. This has enabled us to characterize its interactions with subtilisin in the folding reaction by spectroscopic and microcalorimetric methods. These studies focus on (1) the physical nature and energetics of propeptide interactions with native subtilisin and (2) how the propeptide acts to catalyze folding.

A model for the propeptide–subtilisin folding reaction can be described as the following equilibrium:

$$P_u + S_u \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} P\text{–}S \underset{k_{-2}}{\overset{k_2}{\Leftrightarrow}} P_f\text{–}S_f \underset{k_{on}}{\overset{k_{off}}{\Leftrightarrow}} P_u + S_f$$

where P_u is propeptide and S_u is subtilisin, which are largely unstructured at the start of the reaction; P–S is a collision complex of subtilisin and propeptide; P_f–S_f is the complex of folded subtilisin and propeptide; and S_f is folded subtilisin. In this reaction scheme, the P_u is acting as an enzyme in the folding of subtilisin. The goal of this series of experiments is to characterize all the steps in this reaction. The first step will be to characterize the binding of propeptide to folded subtilisin. The second step will be to determine the energetics of subtilisin folding in the presence of the propeptide.

Since unfolded forms of subtilisin and propeptide are very sensitive to proteolysis, it was necessary to decrease the native proteolytic activity of subtilisin. This was accomplished by

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[1] The specific activities of all S221C mutants discussed here are similar against the synthetic substrate, sAAPFna. (S.A. $\sim$ 0.0025 unit/mg at 25 °C, pH 8.0).

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Table I: Subtilisin Mutations[a]

	S221C	Δ75–83	N218S	M50F	Y217K
S12	+	–	+	+	+
S15	+	+	+	+	+

[a] The plus signs show that a subtilisin contains a particular mutation. The wild-type numbering of amino acids is used for simplicity in defining mutations even when the nine amino acids (75–83) have been deleted. X-ray crystal structures of S12 and S15 have been determined to be 1.8 Å (Bryan et al., 1992; Gallagher et al., 1993).

converting the active-site serine 221 to cysteine by site-directed mutagenesis.[1] This mutation reduces peptidase activity to a level that is measurable but no longer problematic for folding studies. The S221C mutant has become a vehicle for all of our characterizations of the folding and unfolding reactions (Bryan et al., 1992).[2]

Previous efforts to understand the physical and energetic nature of the kinetic barriers to subtilisin folding have established that the high-affinity calcium site-A imposes a large enthalpic barrier to folding and unfolding (Bryan et al., 1992). Removing the calcium site-A from subtilisin by deleting amino acids 75–83 enormously accelerates both the unfolding and refolding reactions. In this report we investigate the interactions of the propeptide with two S221C subtilisins: one with the native calcium A-site (S12) and one without (S15). The mutations in S12 and S15 subtilisins are described in Table I.

MATERIALS AND METHODS

Cloning, Mutagenesis, and Expression of Subtilisin Mutants. The subtilisin gene from *Bacillus amyloliquefaciens* (subtilisin BPN′) has been cloned, sequenced, and expressed at high levels from its natural promoter sequences in *Bacillus subtilis* as previously described (Bryan, 1992; Vasantha et al., 1984; Wells et al., 1983).

Mutant genes for S12 and S15 (Table I) were recloned into a pUB110-based expression plasmid and used to transform *B. subtilis*. The *B. subtilis* strain used as the host contains a chromosomal deletion of its subtilisin gene and therefore produces no background wild-type activity. Oligonucleotide mutagenesis was carried out as previously described (Bryan et al., 1986). S12 and S15 subtilisins were expressed in a 1.5 l New Brunswick fermenter at a level of ~150 mg of the correctly processed mature form/L. The addition of wild-type subtilisin to promote production of the mature form of S221C subtilisin was not required in our bacillus host strain as was the case for Abrahmsen et al. (1991). Variant S221C subtilisins S15 and S12 were purified and verified for homogeneity essentially as described (Bryan et al., 1986; Pantoliano et al., 1987, 1988).

Cloning of the Propeptide of Subtilisin. The propeptide region of the subtilisin BPN′ gene was subcloned using the polymerase chain reaction in an Eppendorf MicroCycler according to conditions outlined in the GeneAmp PCR reagent kit. Oligonucleotides were synthesized which amplified the coding sequence for two versions of the propeptide: the 77 amino acid version, corresponding to the normal C-terminal cleavage site, and an 81 amino acid version which includes the first four amino acids of mature subtilisin (AQSV) at the C-terminus. Digestion of the amplified product with the appropriate restriction enzymes allowed a precise excision of the DNA that codes for either the 77 or 81 amino acid propeptide which was precisely fused to the ATG initiation codon of an *Escherichia coli* expression plasmid with an IPTG-inducible promoter.[3] The strategy is identical to that described in detail for high-level production of the 56 amino acid protein G, B-domain (Alexander et al., 1992a).

Fermentation and Expression of Propeptide. The *E. coli* production strain was grown at 37 °C in a 1.5-L BioFlo Model fermenter until an A600 1–1.5 was attained, at which time 1 mM IPTG was added to induce the production of T7 RNA polymerase that directs synthesis of target DNA message (Alexander et al., 1992). Two hours after induction the cells were harvested.

Protein Purification of Propeptide. *E. coli* paste from a 1.5-L fermentation (5 g) was suspended in 50 mL of cold phosphate-buffered saline (PBS), and PMSF was added to a final concentration of 1 mM. DNase I (1 mg) in 2 mL of 40 mM Tris-HCl and 1 M $MgCl_2$ was also added. This suspension was heated to 80 °C for 5 min. After the reaction was cooled on ice, another addition of PMSF and DNase I was made. This mixture was centrifuged at 25000*g* for 30 min (Alexander et al., 1992a).

The soluble, heat-released protein was dialyzed extensively against 20 mM HEPES, pH 7.0, and purified to homogeneity by anion-exchange chromatography with DE52 followed by cation-exchange chromatography using SE53. Five grams of *E. coli* paste yields 30 mg of purified propeptide.

N-Terminal Analysis. The first five amino acids of the purified propeptide were determined by sequential Edman degradation and HPLC analysis. This revealed that >95% of the purified material had the amino acid sequence expected from the DNA sequence of the gene (N-terminal sequence of AGKSN). The N-terminal methionine synthesized in the *E. coli* production system was absent in the final product. A 56 amino acid propeptide breakdown product was also purified. The N-terminus of this fragment begins at the sequence SAAKK.

Determination of Extinction Coefficient. The propeptide has four tyrosines and no tryptophans. Since the peptide is disordered at low ionic strength, the extinction of free tyrosine (ϵ = 1413 M^{-1}) was used to estimate the extinction of propeptide (5650 M^{-1}). The molecular weight of the 77 amino acid propeptide is 8475. This yields A_{275} of 0.67 = 1 mg/mL.

Titration Calorimetry Measurements. Calorimetric titrations were performed with a Microcal Omega titration calorimeter as described in detail by Wiseman et al. (1989). The titration calorimeter consists of a matched reference cell containing the buffer and a solution cell (1.374 mL) containing the subtilisin solution. Aliquots of the pro solution can be added to the cell through a rotating stirrer syringe operated with a plunger driven by a stepping motor. The accompanying heat change per injection is determined by a thermoelectric sensor between the cells. The area of each peak represents the amount of heat accompanying binding of the added ligand to the protein. The total heat, Q, is then fitted by a nonlinear least squares minimization method (Wiseman et al., 1989) to

[2] A shorthand for denoting amino acid substitutions employs the single-letter amino acid code as follows: Y217K denotes the change of Tyr 217 to Lys.

[3] Abbreviations: CD, circular dichroism; Δ75–83 subtilisin, subtilisin BPN′ with a deletion of amino acids 75–83; cps, counts per second; EDTA, disodium salt of ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; K_a, association constant for propeptide binding; [P], propeptide concentration; [S], subtilisin concentration; sAAPFna, succinyl-(L)-Ala-(L)-Ala-(L)-Pro-(L)-Phe-*p*-nitroanilide; Tris, tris(hydroxymethyl)aminomethane; $t_{1/2}$, half-life for a kinetic experiment.

the total ligand concentration, $[P]_{total}$, according to the equation:

$$dQ/d[P]_{total} = \Delta H[1/2 + (1 - (1 + r)/2 - X_r/2)/ (X_r - 2X_r(1 - r) + 1 + r^2)^{1/2}] \quad (1)$$

where $1/r = [S]_{total} \times K_a$ and $X_r = [P]_{total}/[S]_{total}$.

The titration calorimeter is sensitive to changes in K_a under conditions at which the product of $K_a \times$ [subtilisin] is between 1 and 1000 (Wiseman et al., 1989). The subtilisin concentrations ranged from 15 to 25 μM while the concentrations of the propeptide solutions were about 10–20 × the protein concentrations. Each binding constant and enthalpy were based on two titration runs at each temperature. Titration runs were performed until the titration peaks were close to the base line.

Kinetics of Subtilisin Folding. For refolding studies subtilisin was maintained as a stock solution in 5 mM HEPES, pH 7.5, at a concentration of ~100 μM. To measure subtilisin renaturation rates as a function of propeptide concentration, subtilisin was denatured by mixing 25 μL of protein solution with 12.5 μL of 5 M HCl in a total volume of 100 μL (pH ~1.8). The final HCl concentration was 0.625 M. The Δ75–83 subtilisin mutant S15 is completely denatured in less than 1 s by these conditions (Bryan et al., 1992). Complete denaturation of S12 subtilisin requires 1 h. The rate of denaturation was determined by the 15% decrease in intrinsic tryptophan fluorescence (excitation λ = 300 nm, emission λ = 345 nm), which occurs upon unfolding of subtilisin. The completeness of denaturation was also verified by comparing the far-UV CD spectra of the proteins at pH 1.8 with their spectra in 6 M guanidine hydrochloride, pH 7.0.

Acid-denatured S15 protein was neutralized after 5 s by diluting 100 μL of denatured protein to 2.5 mL in 30 mM Tris base and 5 mM KPO_4, with rapid stirring (final pH = 7.5). Acid-denatured S12 protein was neutralized in the same manner but after 1 h. The desired concentration of propeptide was then added. The final concentration of subtilisin was 1 μM.

The rate of renaturation was determined by an increase in intrinsic tryptophan fluorescence of 1.7-fold (excitation λ = 300 nm, emission λ = 345 nm), which occurs upon folding of subtilisin into a complex with propeptide. Data were obtained using a SPEX FluoroMax spectrofluorimeter for manual mixing experiments. Rates determined by fluorescence increase correlate exactly with rates measured by CD (i.e., the increase in negative ellipticity at λ = 222 nm). The CD spectra of renatured S15 subtilisin complexes were compared to the spectra of native subtilisin plus propeptide and found to be identical. Thermal denaturation profiles of renatured and native S15 complexes, monitored by CD (λ = 222 nm) or tryptophan fluorescence, were also identical. In 30 mM Tris-HCl, 5 mM KPO_4, pH 7.5, and 15 μM propeptide, the melting of the complex is biphasic with a small transition at ~55 °C and the main transition at 64 °C.

Kinetics of Binding the Propeptide to Folded Subtilisin. The rate of folding of the propeptide in the presence of folded S15 or S12 subtilisin was monitored by fluorescence using a KinTek stopped-flow Model SF2001. The reaction was followed by the 1.2-fold increase in the tryptophan fluorescence of subtilisin upon its binding of the propeptide. Propeptide solutions of various concentrations in 0.1 M KPO_4, pH 7.0, were mixed with an equal volume of 0.67 μM subtilisin, and 0.1 M KPO_4, pH 7.0, in a single mixing step.

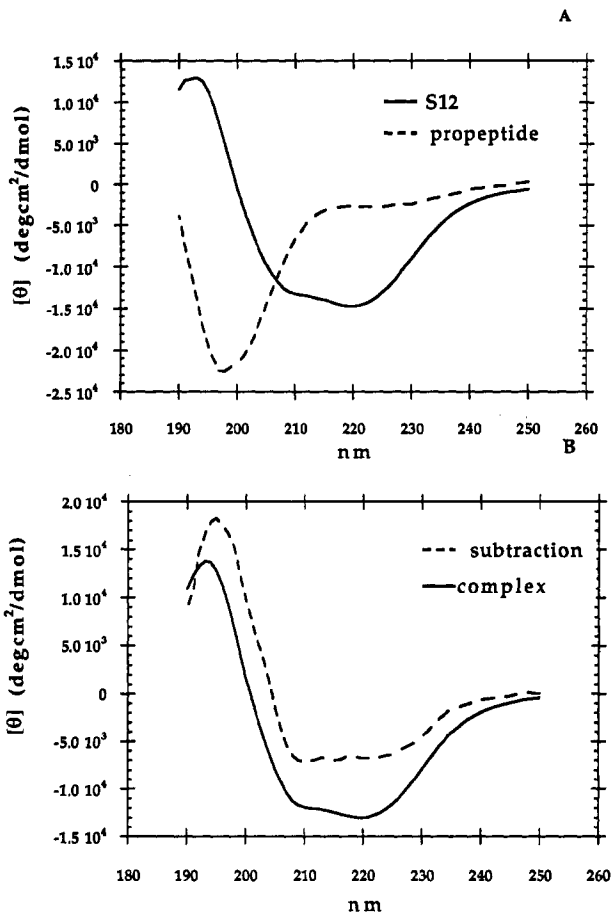

FIGURE 1: Circular dichroism spectra of subtilisin–propeptide complex. (A) Spectra of 100 μM S12 subtilisin and 100 μM isolated propeptide are shown. (B) Spectrum of 100 μM propeptide–S12 complex is shown with a subtracted spectrum of 100 μM propeptide–S12 complex minus 100 μM S12 subtilisin. All spectra were measured in 0.1 M KPO_4, pH 7.0, using a 0.05-mm cylindrical cuvette. Mean residue ellipticity is plotted versus wavelength.

RESULTS

Structure of the 77 Amino Acid Propeptide

The circular dichroism (CD) spectra of S12 subtilisin and the isolated propeptide in 0.1 M KPO_4, pH 7.0, are shown in Figure 1A. The spectrum of the isolated propeptide is typical of a largely random coil structure with a minimum ellipticity at 198 nm. To gain information about the native structure of the propeptide, S12 subtilisin and propeptide were mixed to a final concentration of 100 μM of each. The CD spectrum of the resulting complex is shown in Figure 1B. If no changes in the native subtilisin structure are induced by propeptide binding, then the difference spectrum of the complex minus S12 subtilisin will correspond to the structure of the bound propeptide. Comparison of the subtracted CD spectrum to reference spectra indicates that, in the complex, the propeptide acquires regular secondary structure. Further evidence that the propeptide goes from a disordered to highly ordered state is provided by calorimetric studies described below in detail.

Energetics of Propeptide Binding to Folded Subtilisin

$$P_u + S_f \overset{K_a}{\Leftrightarrow} P_f\text{–}S_f$$

Calorimetric Studies. Titration calorimetry was used to determine thermodynamic state functions ΔG, ΔH, ΔS, and ΔC_p for the reaction of the disordered propeptide with folded

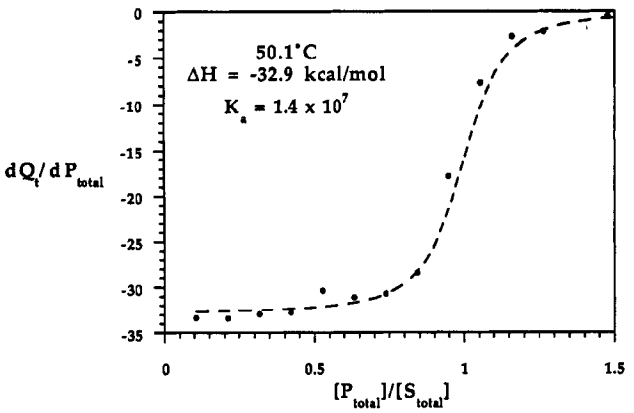

FIGURE 2: Titration calorimetry of subtilisin S12 with isolated propeptide. The heats of binding for successive additions of propeptide are plotted vs the ratio of [pro]/[S12]. The data are best fit by a calculated binding curve assuming a binding constant of 1.4×10^7 and ΔH equal to −32.9 kcal/mol using eq 1 from the text. In this titration [S12] = 15 μM in 0.1 M KPO_4. Temperature was 50.1 °C.

subtilisin. This experiment is analogous to the calorimetric studies performed on the S-peptide of ribonuclease A in complex with the S-protein (Connelly et al., 1990; Varadarajan et al., 1992).

Titrations of S12 and S15 were performed at protein concentrations [S] = 15 and 25 μM and over the temperature range of 25–50 °C. Titration of the S12 subtilisin with propeptide at 50 °C is shown in Figure 2. The data points correspond to the negative heat of binding associated with each addition of propeptide. The titration calorimeter is sensitive to changes in K_a under conditions at which the product of $K_a \times$ [S] is between 1 and 1000 (Wiseman et al., 1989). Since the K_a of S12 subtilisin for propeptide is about 1×10^7 M^{-1} at 50 °C, these protein concentrations result in values of $K_a \times$ [S] = 150 and 250. S12 subtilisin is fully native throughout the temperature range of 25–50 °C. Titrations of S15 subtilisin were not carried out above 37 °C because of its lower stability (Bryan et al., 1992). From the temperature dependence of ΔH, the heat capacity change (ΔC_p) upon binding was determined to be −1.03 kcal/(deg·mol) from the equation:

$$\Delta H = \Delta H_0 + \Delta C_p(T - T_0) \quad (2)$$

The plot of ΔH vs temperature is shown in Figure 3A.

The binding parameters obtained for S12 and S15 are similar: $\Delta H = \sim -7.5$ kcal/mol, $\Delta C_p \sim 1$ kcal/(deg·mol), and $K_a \sim 2 \times 10^8$ M^{-1} at 25 °C, and a stoichiometry of binding of 1 propeptide per subtilisin. The results of titrations of S12 and S15 are summarized in Table II.

Association constants for propeptide binding to S12 subtilisin were determined by curve fitting the data at temperatures of 40, 45, and 50 °C using eq 1. At the lower temperatures the smaller amount of heat produced per titration, coupled with tighter binding, makes direct determination of association constants less accurate. Nevertheless, since ΔH at a reference temperature and ΔC_p are known, the equilibrium constant can be calculated as a function of temperature from the equation:

$$K_{eq} = K_{eq}^0\{\exp[(-\Delta H_0/R)(1/T - 1/T_0) + (\Delta C_p T_0/R)(1/T - 1/T_0) + (\Delta C_p/R)\ln(T/T_0)]\} \quad (3)$$

where K_{eq}^0 is the equilibrium constant at T_0 (Brandts & Lin, 1990; Connelly et al., 1990). A profile of K_a vs T for propeptide binding was calculated from eq 3, using the experimentally determined K_a's at 40, 45, and 50 °C and $\Delta C_p = -1.03$ kcal/(deg·mol) (Figure 3B). The calculated curve is analogous to a stability curve for protein denaturation (Becktel & Schellman, 1987). The maximum K_a of the complex (2.3×10^8 M^{-1}) occurs at ~20 °C. At this temperature $\Delta H = 0$ and binding energy is entirely due to a positive entropy change of 38 cal/(deg·mol). The net positive entropy change upon binding presumably results from the large gain of entropy from hydrophobic burial exceeding the loss of conformational entropy. The overall thermodynamics of propeptide binding to folded subtilisin are typical of the folding reaction of other small globular proteins (Becktel et al., 1987; Privalov, 1979).

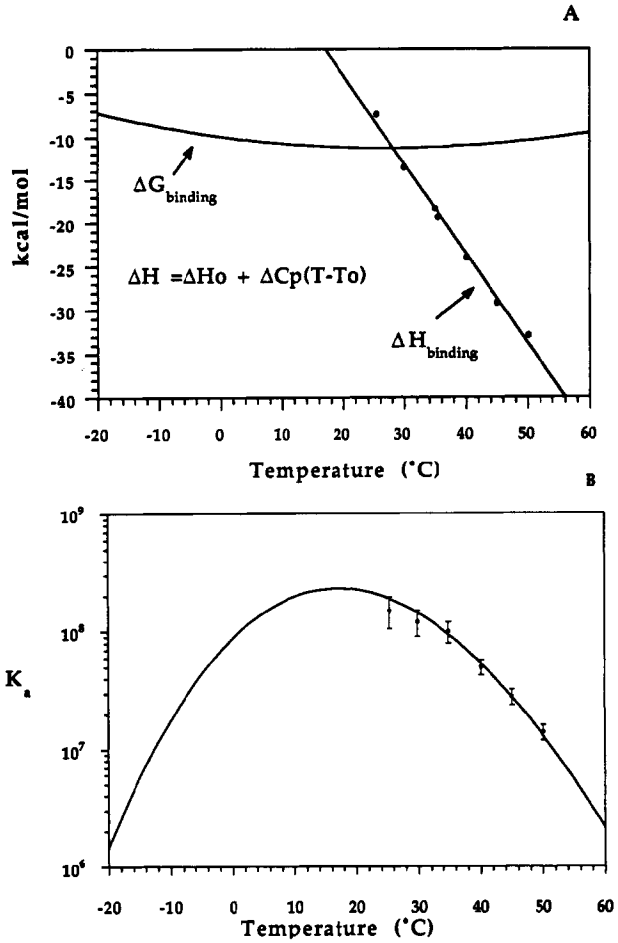

FIGURE 3: Energy of binding propeptide to S12 subtilisin vs temperature. (A) A plot of ΔH vs temperature is linear over the range of 25–50 °C and allows determination of the heat capacity change upon binding from eq 2 in the text ($\Delta C_p = -1.03$ kcal/mol). The ΔG curve is calculated from eq 3 in the text. (B) The temperature dependence of K_a of the propeptide–S12 complex is shown. The K_a's were determined by curve fitting titration data using eq 1 as described in the text. The curve shown above is calculated from eq 3, using $\Delta C_p = -1.03$ kcal/(deg·mol).

Kinetics of Binding the Propeptide to Folded Subtilisin

$$P_u + S_f \underset{k_{off}}{\overset{k_{on}}{\Leftrightarrow}} P_f\text{–}S_f$$

The kinetics of folding the propeptide in the presence of native subtilisin were measured by stopped-flow mixing methods. The reaction was followed by the increase in tryptophan fluorescence upon binding. The purpose of the experiment was to measure the second-order rate constant, k_{on}, for binding propeptide to folded subtilisin. From this measurement, the off-rate can be determined using the equilibrium constant for binding from the titration calorimetry

Table II: Titration Calorimetry of Subtilisin Mutants S12 and S15 with Propeptide

		parameters calculated from fit			
[S]	T (°C)	n	K_a (M^{-1})	ΔH (kcal/mol)	ΔC_p [kcal/(deg·mol)]
S12, 15 μM	25.4	0.95 ± 0.03	$\sim 10^8$	−7.4 ± 0.5	
	29.9	0.96 ± 0.02	$\sim 10^8$	−13.6 ± 0.2	
	34.9	0.92 ± 0.05	$\sim 10^8$	−18.4 ± 0.3	
	40.1	0.97 ± 0.02	$(5.0 \pm 1.5) \times 10^7$	−24.0 ± 0.3	
	45.1	0.97 ± 0.04	$(2.8 \pm 1.0) \times 10^7$	−29.2 ± 0.3	
	50.1	0.95 ± 0.03	$(1.4 \pm 0.5) \times 10^7$	−32.9 ± 0.4	
					−1.03
S15, 25 μM	25.4	0.85 ± 0.05		−7.5 ± 1.2	
	36.2	0.82 ± 0.03	(5.0 ● 2.5) × 10^7	−22.7 ± 0.2	
					−1.0

[a] Binding parameters for stoichiometric ratio (n), binding constant (K_a), and binding enthalpy (ΔH) were determined using nonlinear least-squares minimization of the titration data to eq 1 in the text (Wiseman et al., 1989). Measurements for each experimental condition were performed in duplicate at each temperature. K_a's at the lower temperatures cannot be determined precisely by curve fitting due to the decreasing enthalpy of binding. At 20 °C the ΔH is ~0.

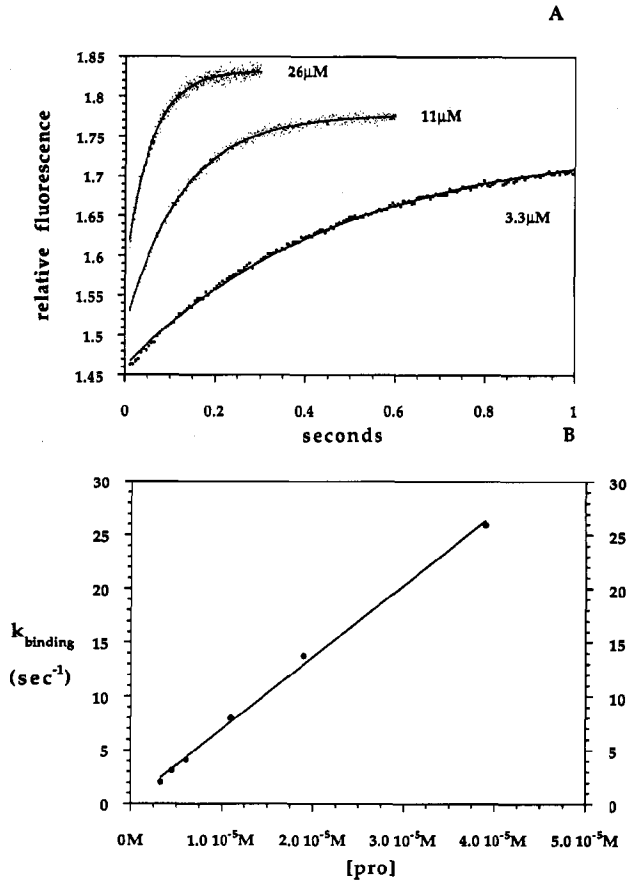

FIGURE 4: Folding rate of the propeptide in the presence of folded S12 subtilisin. (A) Folding was monitored by fluorecence using a KinTek stopped-flow Model SF2001. Propeptide solutions of various concentrations in 0.1 M KPO_4, pH 7.0, were mixed with an equal volume of 0.67 μM S12 subtilisin and 0.1 M KPO_4, pH 7.0, in a single mixing step. The final propeptide concentrations are indicated on the graph. The reaction was followed by the 1.2-fold increase in tryptophan fluorescence, which occurs upon formation of the propeptide–subtilisin complex. The data are fit to a single exponential equation to determine a pseudo-first-order rate constant for folding (solid lines). Temperature was 25 °C. (B) The pseudo-first-order rate constant for folding and binding, $k_{observed}$, is plotted as a function of $[P_u]$. Over the 10-fold range of $[P_u]$, $k_{observed}$ was a linear function of $[P_u]$ and could be fit to the equation: $k_{observed} = 0.27 + (6.74 \times 10^5)[P_u]$.

experiments. The propeptide does not contain tryptophan and has no intrinsic fluorescence at 345 nm if an excitation wavelength of 300 nm is used, as in these experiments. Subtilisin contains three tryptophans. Fluorescence at 345 nm increases by 1.2-fold due to the change in environment of tryptophans in S_f upon the binding of propeptide. If the reaction is carried out with a 10-fold or greater excess of P_u, then one observes a pseudo-first-order kinetic process with a rate of $k_1[P_u]$. The folding reaction was followed using $[S_f] = 0.33$ μM and varying $[P_u]$ from 5 to 50 μM (Figure 4A). The $k_{observed}$ was plotted as a function of $[P_u]$. Over the 10-fold range of $[P_u]$, $k_{observed}$ was a linear function of $[P_u]$ and could be fit to the equation: $k_{observed} = 0.27 + (6.74 \times 10^5)[P_u]$ (Figure 4B). The absence of noticeable curvature in the plot implies that the isomerization of the collision complex of P_u and S_f is rapid relative to its formation. The second-order rate constant $k_{on} = 6.74 \times 10^5$ M^{-1} s^{-1}. The y-intercept in this plot is equal to the off-rate, k_{off}. The value determined from this plot is imprecise, however, because the off-rate is small. The off-rate can be better estimated using the relationship $K_a = k_{on}/k_{off}$. The calorimetrically determined association contant for the complex at 25 °C, 1.9×10^8 M^{-1}, yields k_{off} equal to 0.0035 s^{-1}.

Energetics of Propeptide-Catalyzed Subtilisin Folding

$$P_u + S_u \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P\text{–}S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P_f\text{–}S_f$$

Kinetic Measurements. The folding reaction of subtilisin, in the presence of propeptide, can be followed by an increase in tryptophan fluorescence of 1.7-fold due to changes in the environments of the three tryptophans in subtilisin upon its folding and binding of the propeptide. As described above, the propeptide does not contain tryptophan and has no intrinsic fluorescence at 345 nm if an excitation wavelength of 300 nm is used. Therefore fluorescence increases observed at 345 nm are due to the conversion of S_u to P_f–S_f.

We attempted to follow the propeptide-catalyzed folding reactions of subtilisin S12 and S15 (the Δ75–83 version). No significant folding of 1 μM S12 subtilisin could be measured over a time scale of hours, even in the presence of 100 μM propeptide. We believe that covalent attachment of the propeptide to subtilisin may be required to avoid a kinetic trap involving premature folding of the high-affinity A-site region, as discussed below. In contrast, the folding of Δ75–83 subtilisin is efficiently catalyzed by the detached propeptide.

Folding experiments for S15 subtilisin were carried out at lower ionic strength than the binding studies to decrease the rate of uncatalyzed folding. In 0.1 M KPO_4, the uncatalyzed rate of S15 folding is 0.002 s^{-1} at 25 °C. At 30 mM Tris and 5 mM KPO_4, pH 7.5, S15 folds at a rate of $\leq 6 \times 10^{-5}$ s^{-1} at 25 °C (Figure 5A). The rate of folding in either case is accelerated as a function of $[P_u]$. The folding reaction at low

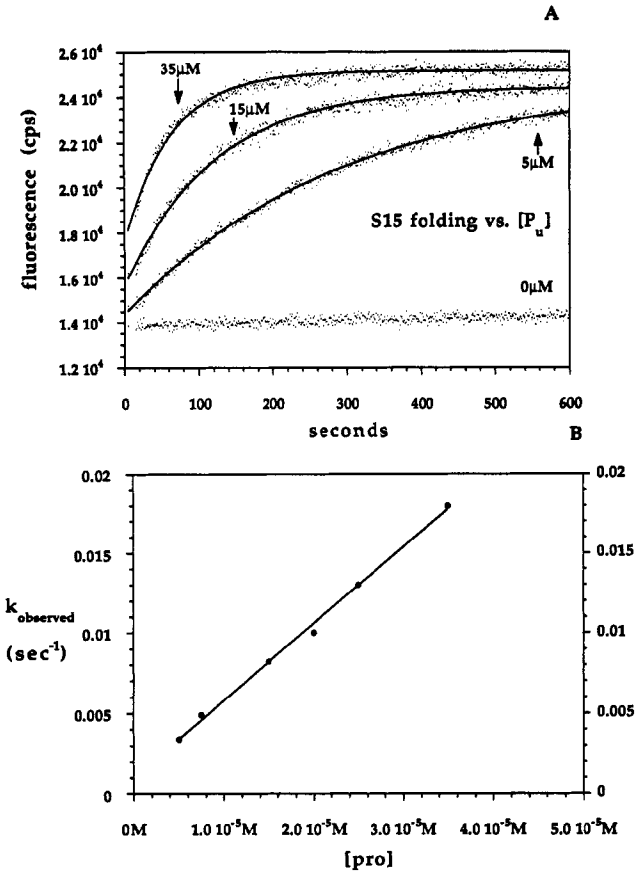

FIGURE 5: Folding rate of S15 subtilisin in the presence of the propeptide. (A) Folding was monitored by fluorescence using a SPEX Fluoromax. Propeptide solutions of various concentrations and 1 μM denatured S15 subtilisin were mixed in 5 mM KPO_4 and 30 mM Tris, pH 7.5. The final propeptide concentrations are indicated on the graph. The reaction was followed by the 1.7-fold increase in tryptophan fluorescence, which occurs upon folding of subtilisin into the propeptide–subtilisin complex. The data are fit to a single exponential equation to determine a pseudo-first-order rate constant for folding (solid lines). Temperature was 25 °C. (B) The pseudo-first-order rate constant for folding, $k_{observed}$, is plotted as a function of $[P_u]$. Over the 10-fold range of $[P_u]$, $k_{observed}$ was a linear function of $[P_u]$ and could be fit to the equation: $k_{observed} = 0.0011 + 478[P_u]$.

ionic strength was followed using $[S_u] = 1$ μM and varying $[P_u]$ from 5 to 35 μM. As described above, the reaction is a pseudo-first-order kinetic process when P_u is in sufficient molar excess of S_u (Figure 5A). The $k_{observed}$ was plotted as a function of $[P_u]$ (Figure 5B). We have modeled the reaction assuming that the formation of the collision complex can be approximated as a rapid equilibrium reaction, with the equilibrium constant, K_1, equal to $[P\text{–}S]/[S_u][P_u]$ (Anderson et al., 1991). The rate of formation of S_f–P_f will depend on the concentration of P–S. Since $[S\text{–}P]/[S_{total}] = K_1[P_u]/(1 + K_1[P_u])$, the observed rate of formation of S_f–P_f would be equal to

$$\{k_2K_1[P_u]/(1 + K_1[P_u])\} + k_{-2} \quad (4)$$

The concentration dependence of the rate should follow a hyperbola, which is a function of the saturation of [S–P] (Anderson et al., 1991). Since the folding of subtilisin in the absence of propeptide is a slow process, we expected that isomerization of the collision complex, P–S, to the fully folded complex, P_f–S_f, might become the rate-limiting step in the reaction at high $[P_u]$. The absence of curvature in the plot implies, however, that the isomerization of P–S is rapid relative to its formation. Fitting the plot of $k_{observed}$ vs $[P_u]$ in Figure 5B to eq 4 indicates that $K_1 \leq 10^3$ and that $k_2 \geq 0.5$ s^{-1}. If

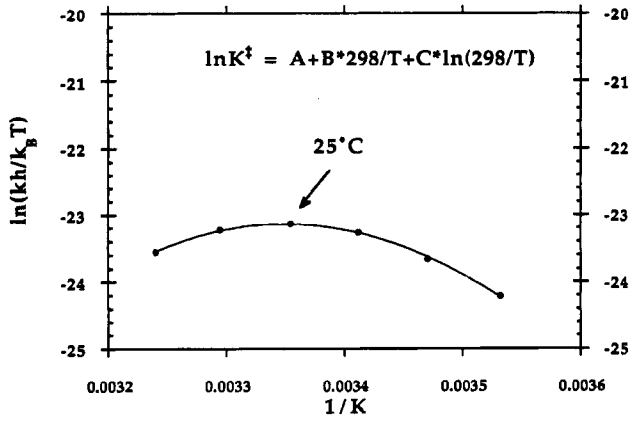

FIGURE 6: Temperature dependence of the refolding rate of S15 subtilisin plus propeptide. The natural log of the equilibrium constant for the transition state (calculated from the Eyring equation) is plotted vs the reciprocal of the absolute temperature. The line is fit according to eq 6 in the text, with $T_0 = 298$ K.

K_1 were as large as 10^4, then the plot would deviate noticeably from linearity at the highest $[P_u]$. The lower limit for K_1 is not known since we were not able to raise $[P_u]$ high enough to saturate the collision complex and cause k_2 to become limiting in the reaction. Over the $[P_u]$ examined here the formation of a productive collision complex is the limiting step in the reaction, and the data can be approximated to the simpler equilibrium:

$$P_u + S_u \underset{k_{unf}}{\overset{k_1}{\Longleftrightarrow}} P_f\text{–}S_f$$

The second-order rate constant $k_1 = 480$ M^{-1} s^{-1}. The reverse rate for unfolding of the complex k_{unf} is equal to the y-intercept in this plot and is obviously very small, though an accurate value cannot be determined from these experiments.

Temperature Dependence of the Catalyzed Folding Reaction

Second-order rate constants (k_1) for folding were measured over the temperature range of 10–35 °C. A plot of ln k_1 vs $1/T$ is not linear, indicating that the folding reaction cannot be described in terms of simple Arrhenius theory. This behavior has been observed previously for the folding reaction of other proteins and is due to the decrease in heat capacity which occurs in going from the unfolded state to the transition state (Chen et al., 1989; Pohl, 1968).

We have fit our data to the Eyring equation:

$$\ln K^* = -RT \ln(kh/k_BT) \quad (5)$$

where k_B is the Boltzman constant, h is Planck's constant, and k is the second-order rate constant for folding of the subtilisin propeptide complex. The graph of $\ln(kh/k_BT)$ vs $1/T$ (K) is shown in Figure 6. The data are fitted to the equation:

$$\ln K^* = A + B(T_0/T) + C \ln(T_0/T)$$

where $A = [-\Delta C_p^* + \Delta S^*(T_0)]/R$; $B = -A - \Delta G^*(T_0)/RT_0$; and $C = -\Delta C_p^*/R$ (Chen et al., 1989). This treatment of the kinetic data is based on vibrational theory in which the frequency of breakdown of the transition state to product is equal to $\kappa k_BT/h$, where κ is a transmission coefficient assumed to be equal to 1. The curvature in the plot is due to the decrease in heat capacity [$\Delta C_p^* = -1.5$ kcal/(deg·mol)] in going from the unfolded state to the transition state. The overall $\Delta C_{p,folding}$ for subtilisin plus propeptide is $\sim$–5 kcal/(deg·mol). The change in heat capacity associated with formation of the

transition state is therefore about 30% of the total heat capacity change for folding. The change in heat capacity upon protein folding is correlated with the change in environment of apolar groups (Livingstone et al., 1991). The maximum rate of catalyzed S15 folding occurs at ~25 °C. The slope of the curve at 25°C is almost zero, indicating that $\Delta H^{\ddagger}$ is small and that the activation barrier to catalyzed folding is almost completely entropic.

Influence of Altered Forms of the Propeptide and SSI on Subtilisin Folding

We have tested two altered versions of the propeptide in the subtilisin folding reaction. A 56 amino acid breakdown product of the propeptide was recovered from the *E. coli* expression strain and purified. This truncated version is missing the N-terminal 21 amino acids and was shown by peptide sequencing to begin with the amino acids SAAKK. A second 81 amino acid version of the propeptide was synthesized intentionally. In this version, the first four residues of mature subtilisin were included at the C-terminal end of the propeptide. Our reasoning was that an independently stable propeptide structure might be achieved if part of the mature subtilisin sequence were included. Neither of these peptides had any secondary structure detectable by CD, however, and neither has any effect on the rate of folding of S12 or S15 subtilisin. We also found that the streptomyces subtilisin inhibitor protein (SSI) has no detectable influence on the rate of S15 folding.

In these experiments, 5 μM of an altered propeptide or SSI was added to 1 μM of unfolded S15 subtilisin in 30 mM Tris and 5 mM KPO_4, pH 7.5, and 25 °C. The reactions were followed by fluorescence for 30 min. The change in fluorescence over this time period was similar to the uncatalyzed reaction rate. After 30 min, 5 μM of the 77 amino acid propeptide was added. Upon the addition of unaltered propeptide, folding of subtilisin occurred at a rate of 0.003 s^{-1}, which is the same rate observed if altered propeptide or SSI is not included in the reaction.

DISCUSSION

Subtilisin has a high activation barrier between folded and unfolded state, so that the in vitro folding reaction is extremely slow. We estimate that the $t_{1/2}$ for in vitro folding of wild-type subtilisin, under optimal conditions, is weeks or longer. The Δ75–83 deletion, which removes the calcium A-site, accelerates uncatalyzed folding by an estimated 10^4- to 10^5-fold (Bryan et al., 1992; Gallagher et al., 1993). Denatured S15 subtilisin, when returned to native conditions at low ionic strength, is kinetically isolated from the native state and remains largely devoid of regular structure for hours. When the isolated and unstructured propeptide is added, folding of both occurs spontaneously. This foldase activity of the propeptide is a specific effect, however, since two altered versions of the propeptide are unable to catalyze the folding of S12 or S15 subtilisin. If the propeptide were a stable structure independent of subtilisin, then it would be easier to understand how it could catalyze folding by binding to and stabilizing a native-like transition state, as is proposed to be the case for α-lytic protease and its propeptide (Baker et al., 1992). The reaction of unfolded propeptide with unfolded S15 subtilisin to form a stable, folded complex is an intriguing case of complementation. On the basis of the results reported here, the energetics of the folding reaction of S15 subtilisin and propeptide can be summarized as follows:

$$P_u + S_u \underset{k_{-1}\ \geq 0.5\ s^{-1}}{\overset{k_1\ 480\ M^{-1}\ s^{-1}}{\rightleftharpoons}} P\text{–}S \underset{k_{-2}\ <0.001\ s^{-1}}{\overset{k_2\ \geq 0.5\ s^{-1}}{\rightleftharpoons}} P_f\text{–}S_f \underset{k_{on}\ 7\times10^5\ M^{-1}\ s^{-1}}{\overset{k_{off}\ 0.0035\ s^{-1}}{\rightleftharpoons}} P_u + S_f$$

The rate-limiting step in the folding of S15 subtilisin is formation of the initial collision complex, P–S. A diffusion-controlled reaction involving macromolecules can occur at a rate of 10^9 M^{-1} s^{-1}. The rate at which P_u and S_u form a productive collision complex is very infrequent, however, with $k_1 \sim 500$ M^{-1} s^{-1}. The kinetic results indicate that once a productive collision complex is formed, subsequent isomerization is rapid. The rate constant for isomerization of the collision complex to the folded complex is not known but must be ≥0.5 s^{-1} in order for isomerization not to become the rate limiting step in folding at $[P_u] = 35$ μM.

Formation of a productive collision complex may require that a partially structured subtilisin molecule collide with structured propeptide, thus providing a nucleation point for subsequent folding. The extremely slow rate of uncatalyzed S15 folding at low ionic strength indicates that the probability of forming productive folding intermediates in subtilisin alone is low. CD experiments indicate that the equilibrium constant for the $P_u \Leftrightarrow P_f$ transition for isolated propeptide is also very small. We suspect that the collision complex is a stable early folding unit resulting from the collision of short-lived species of subtilisin and propeptide. This mechanism would explain why the second-order rate constant for folding S15 subtilisin with the detached propeptide is small. Reaction rate analysis shows that the activation barrier for forming the productive collision complex is almost entirely entropic at 25 °C (the temperature at which the catalyzed folding rate is fastest).

The change in heat capacity associated with formation of the transition state for catalyzed folding is consistent with the idea that the collision complex is an early folding unit. $\Delta C_p^{\ddagger}$ is about 30% of the total heat capacity change for folding. Since $\Delta C_{p,\text{folding}}$ is correlated with the change in environment of apolar groups, the transition state for the catalyzed folding reaction of subtilisin appears to be more similar in compactness to the unfolded state than to the native state. This result contrasts with observations made for the folding reactions of several smaller globular proteins. For example, $\Delta C_p^{\ddagger}$ of T4 lysozyme (Chen et al., 1989), chymotrypsin inhibitor 2 (Jackson & Fersht, 1991), and protein G, B domain (Alexander et al., 1992b), are all ~70% of the total ΔC_p for folding.

Structural complementarity of folded propeptide and folded subtilisin is evidenced by a K_a of the complex equal to 2.3×10^8 M^{-1} at 20 °C. The thermodynamic state functions for binding the propeptide to folded subtilisin are typical of the folding reaction of a small globular protein. The energetics of binding the propeptide to the folded forms of either S15 or S12 subtilisin are similar. Folding of S12 subtilisin, however, is extremely slow even in the presence of a high concentration of isolated propeptide. The high K_a of the complex means that recycling of the propeptide to fold additional subtilisin molecules is very slow in our in vitro reaction. Given the tight binding of propeptide to subtilisin, one wonders how mature subtilisin is released after processing and secretion in vivo. In vivo, additional proteolytic steps on the propeptide might be required after the initial processing step to disrupt the propeptide–subtilisin complex.

Why Doesn't the Detached Propeptide Fold Subtilisin with the Native Calcium A-Site? We have not been able to conclusively demonstrate folding of subtilisin S12, which has

the wild-type calcium A-site, even in the presence of 100 μM propeptide. It has been recently reported that a small percentage of native subtilisin is recovered in the presence of isolated propeptide after 8 days (Eder et al., 1993). Folding on this time scale would be unlikely to be of biological relevance, however. We believe that covalent attachment of the propeptide to subtilisin may be required to avoid a kinetic trap involving premature folding of the high-affinity A-site region. Eder et al. have reported that subtilisin without propeptide folds to a highly structured intermediate state which binds calcium. This partially folded state may serve as the kinetic trap, which blocks subsequent folding to the native state. The covalently attached propeptide may prevent the formation of this intermediate by dictating the sequence of folding events, such that the A-site region is folded late in the pathway.

The mechanism of processing the propeptide is consistent with this idea. In vivo processing is known to be autocatalyic, although it is uncertain whether it occurs intramolecularly or intermolecularly (Ikemura et al., 1987; Power et al., 1986). In either case, the N-terminus of mature subtilisin must occupy the substrate binding pocket of a subtilisin molecule for processing to occur. Since the N-terminal four amino acids of the mature subtilisin are involved in β-pair hydrogen bonds with the calcium binding loop and the side-chain carbonyl oxygen of Q2 is a ligand to the calcium in the A-site, it is hard to imagine how the N-terminus of completely native subtilisin could bind in the P1′ pocket of the subtilisin active site. Thus the covalent attachment of the propeptide to mature subtilisin may prevent the final folding of the calcium A-site region, until after its cleavage to produce the mature enzyme. This argument is supported by two experimental observations. First, the folding of Δ75–83 subtilisin, which lacks the A-site, is efficiently catalyzed by the detached propeptide. Second, the binding affinities of the propeptide for S12 and S15 subtilisins are similar, indicating that a native A-site is not part of the recognition surface of the propeptide. Purification of large quantities of the intact 352 amino acid prosubtilisin should allow the energetics of folding subtilisin with the native A-site to be better understood.

In summary, we have defined the overall energetics of interactions of the isolated 77 amino acid propeptide with subtilisin. The results suggest a general mechanism for a catalyzed protein folding reaction. Unfolded S15 subtilisin when returned to native conditions at low ionic strength is kinetically isolated from its native state. Upon the addition of isolated and unfolded propeptide, the two cooperate in folding, forming a simple self-assembly system.

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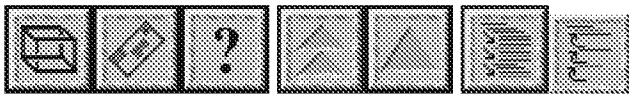
APPENDIX B

Structural Classification of Proteins



Superfamily: Subtilisin-like

Lineage:

1. Root: scop
2. Class: Alpha and beta proteins (a/b) [51349]
   Mainly parallel beta sheets (beta-alpha-beta units)
3. Fold: Subtilisin-like [52742]
   3 layers: a/b/a, parallel beta-sheet of 7 strands, order 2314567; left-handed crossover connection between strands 2 & 3
4. Superfamily: Subtilisin-like [52743]

Families:

1. Subtilases [52744] (18)
2. Serine-carboxyl proteinase, SCP [52764] (3)
   elaborated with additional structures

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